LIMNOLOGY
and
OCEANOGRAPHY

ASLO

Limnol. Oceanogr. 60, 2015, 1588–1603

doi: 10.1002/lno.10119

The contribution of inorganic and organic nutrients to the growth of a North American isolate of the mixotrophic dinoflagellate, *Dinophysis acuminata*

Theresa Hattenrath-Lehmann, Christopher J. Gobler*
School of Marine and Atmospheric Sciences, Stony Brook University, Southampton, New York

Abstract

Diarrhetic Shellfish Poisoning (DSP) is a globally significant human health syndrome most commonly caused by dinoflagellates of the genus *Dinophysis*. While ecosystem studies suggest that blooms of this mixotrophic dinoflagellate can be promoted by excessive nitrogen (N) loading, it is unclear whether these effects are direct (nutrient stimulation of *Dinophysis*) or indirect (nutrient stimulation of prey) since this alga is mixotrophic and culture studies investigating the effects of nutrients on *Dinophysis* have not been performed. We established an isolate of *Dinophysis acuminata* from New York waters and conducted controlled culture experiments to assess the effects of nutrients on the growth of this dinoflagellate with and without its prey, *Mesodinium rubrum*. *Dinophysis* was found to rapidly assimilate ^{15}N-labeled ammonium and urea, AND to a far lesser extent nitrate. Cultures grown with and without prey generally grew faster with ammonium, glutamine, or organic matter from sewage effluent added than respective controls, while nitrate grown cultures yielded significantly more rapid growth only when fed copious amounts of *Mesodinium*. Growth rates of *Dinophysis* also increased with the amount of *Mesodinium* available in cultures and achieved maximal growth rates (0.36 ± 0.01 d^{-1}) when grown with high levels of *Mesodinium* and the amino acid, glutamine. Collectively, this study demonstrates that inorganic and organic N can directly promote the growth of *Dinophysis* and supports the hypothesis that accelerated N loading in coastal ecosystems can promote DSP producing blooms of this species and, thus, may be partly responsible for their recent expansion across the North America.

Diarrhetic Shellfish Poisoning (DSP) is a globally significant human health syndrome most commonly caused by dinoflagellates of the genus *Dinophysis* (Hallegraeff 1993; Van Dolah 2000; Reguera et al. 2012). *Dinophysis* spp. synthesize okadaic acid and dinophysistoxins, the causative toxins of DSP, as well as the coeluted pectenotoxins (Lee et al. 1989; Fux et al. 2011) which are not associated with DSP but may promote the formation of tumors in mammals and may be hepatotoxic (Lee et al. 1989; Burgess and Shaw 2001). While DSP is a common occurrence in regions of Europe, South America, and Asia (Hallegraeff 1993; Van Dolah 2000; Reguera et al. 2012), prior to 2008 the U.S. had never experienced a DSP event. In recent years, however, the U.S. has experienced an expansion of *Dinophysis* blooms causing shellfish to accumulate DSP toxins to levels exceeding the USFDA toxicity threshold (160 ng g^{-1} of shellfish tissue) on the East (NY; Hattenrath-Lehmann et al. 2013), West (WA; Trainer et al. 2013), and Gulf coasts (TX; Campbell et al. 2010; Deeds et al. 2010; Swanson et al. 2010). Interestingly, *Dinophysis* spp. were found in these regions prior to the shellfish toxicity events (Freudenthal and Jijina 1988; Dickey et al. 1992; Horner et al. 1997; Trainer et al. 2013), suggesting these DSP-producing blooms have become more intense in recent years.

To date, *Dinophysis* culture research has focused on differences in toxicity and toxin profiles between species and geographic isolates (Hackett et al. 2009; Kamiyama and Suzuki 2009; Fux et al. 2011), toxin production and/or excretion (Nagai et al. 2011; Tong et al. 2011; Smith et al. 2012; Nielsen et al. 2013), the effects of prey concentration (*Mesodinium rubrum*) on growth (Kim et al. 2008; Kamiyama and Suzuki 2009) as well as toxin production (Nielsen et al. 2012), and the effects of light intensity on the growth and/or toxicity of *Dinophysis* (Kim et al. 2008; Tong et al. 2011; Nielsen et al. 2012, 2013). Despite these recent advances, several faucets of *Dinophysis* ecology remain relatively unexplored since it was only recently that *Dinophysis* cultures were established after the development of a three-step culturing process (Park et al. 2006). Specifically, *Dinophysis* sequesters and utilizes plastids (kleptoplastids) from *M. rubrum*

*Correspondence: christopher.gobler@stonybrook.edu

(=*Myrionecta rubra*; Minnhagen and Janson 2006; Wisecaver and Hackett 2010; Minnhagen et al. 2011) which they, in turn, obtain from their cryptophyte prey (Hansen et al. 2012). While *Dinophysis* can survive months without feeding, this obligate mixotroph is unable to survive long-term without light and its prey *M. rubrum* (Kim et al. 2008; Nielsen et al. 2012; Smith et al. 2012). Recent investigations of North American *Dinophysis* isolates were performed at temperatures (4–10°C) that are not within the range of those found during temperate DSP-producing blooms (13–24°C in NY; Hattenrath-Lehmann et al. 2013, 2015) as the prey used were isolated from polar latitudes (Hackett et al. 2009; Tong et al. 2010, 2011; Fux et al. 2011; Smith et al. 2012). Hence, open questions regarding the growth response of *Dinophysis* cultures at temperatures found during North American blooms.

In contrast to many harmful algae, the nutritional ecology of *Dinophysis* is poorly understood. While it has been established that *Dinophysis* is an obligate mixotroph that feeds on the ciliate *M. rubrum*, whether other prey organisms, including bacteria, contributes to *Dinophysis* growth has not been assessed (Reguera et al. 2012). In addition, given the very recent advance in the establishment of *Dinophysis* cultures, nearly all knowledge regarding *Dinophysis* and nutrients has been gleaned from field investigations that have come to contradictory conclusions (Delmas et al. 1992; Giacobbe et al. 1995; Johansson et al. 1996; Koukaras and Nikolaidis 2004; Seeyave et al. 2009; Hattenrath-Lehmann et al. 2015). Specifically, while *Dinophysis* dominated-communities have displayed a high affinity for ammonium (Seeyave et al. 2009) other field studies found no relationship between *Dinophysis* densities and nutrient concentrations (Delmas et al. 1992; Giacobbe et al. 1995; Koukaras and Nikolaidis 2004). Given that recent evidence from field experiments suggests that nitrogenous nutrients (including B-vitamins) either directly or indirectly (via prey) enhance the growth and toxicity of *Dinophysis acuminata* (Hattenrath-Lehmann et al. 2015), culture experiments specifically isolating the effects of nutrients on both autotrophic (direct) and mixotrophic (indirect) cultures are clearly warranted.

In 2013, a *D. acuminata* culture was established from Meetinghouse Creek, a eutrophic, tidal tributary located in the Peconic Estuary, New York, U.S.A., which experiences annual blooms of *Dinophysis*, with densities that can exceed two million cells L^{-1}. The ability of *Dinophysis* to directly assimilate dissolved nitrogenous nutrients was assessed using ^{15}N-labeled compounds. The autotrophic (no prey) and mixotrophic (with *M. rubrum*) growth of *Dinophysis* was assessed over a gradient of prey-to-*Dinophysis* ratios. The effects of multiple inorganic and organic nutrient sources on *Dinophysis* growth rates were examined using both autotrophic (starved) and mixotrophically (fed) conditioned *Dinophysis* cultures. Similarly, cultures grown with and without nutrients were also grown with and without prey to isolate the effects of nutrients on *D. acuminata* when *M. rubrum* is present (mixotrophic) and when it is not (autotrophic). These are the first experiments to isolate the direct and indirect effects of nutrients on *Dinophysis* spp.

Materials and methods

Cultures and culturing conditions

Cultures of the cryptophyte, *Teleaulax amphioxeia* (K-0434, Scandinavian Culture Collection of Algae and Protozoa), and the ciliate, *M. rubrum* (MBL-DK2009), were isolated in 2009 from Helsingør Harbor, Denmark, and were generously provided by P. J. Hansen. *M. rubrum* was provided with *T. amphioxeia* weekly at a ratio of ~ 10 : 1 (prey : predator) and following complete consumption of the cryptophyte were fed to *D. acuminata* isolates weekly at a ratio of ~ 10 : 1 (prey : predator). During May 2013, clonal isolates of *D. acuminata* were established from Meetinghouse Creek, a tidal tributary located in the Peconic Estuary (40°56.314′N, 72°37.119′W), using 12-well culture plates (Corning, Corning, New York, U.S.A.). Sequencing of the mitochondrial cytochrome c oxidase 1 (*cox1*) gene (Raho et al. 2008; Campbell et al. 2010) revealed that *D. acuminata* from Meetinghouse Creek is 100% identical to both *D. acuminata* from Narragansett Bay, Rhode Island, U.S.A. (accession number EU130566) and Northport Bay, New York (Hattenrath-Lehmann et al. 2013). Two viable *D. acuminata* isolates were combined to mimic field populations and grown out in 2 L Pyrex Erlenmeyer flasks for experiments. All cultures were maintained in sterile *f*/2 (-Si) medium (883 μmol L^{-1} nitrate and 36 μmol L^{-1} phosphate; Guillard and Ryther 1962) made from autoclaved and 0.2 μm-filtered aged coastal Atlantic Ocean water (40.7969°N, 72.4606°W) adjusted to 25 psu and kept at 18°C in an incubator with a 14 : 10 h light : dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of ~ 70 μmol quanta m^{-2} s^{-1} to cultures. Consistent with all prior culture studies of this genus, cultures were xenic as in attempts to culture *Dinophysis* we found that antibiotics were lethal to *Dinophysis*.

Growth experiments with varying *Mesodinium* ratios

Growth rates of *D. acuminata* were assessed over a gradient of predator-to-prey (*M. rubrum*) ratios during a month-long experiment. Stock cultures of well-fed *D. acuminata* and *M. rubrum* (*T. amphioxeia* completely consumed) were diluted to appropriate treatment densities in triplicate using *f*/2 (-Si) medium in 500 mL Erlenmeyer flasks and incubated as above. Initial concentrations of 100 *D. acuminata* cells mL^{-1} were established for all treatments and for a no prey control. *M. rubrum* was added at prey : predator ratios of 2 : 1 (200 cells mL^{-1}), 5 : 1 (500 cells mL^{-1}) and 10 : 1 (1,000 cells mL^{-1}) with additional no predator controls established for each ratio. Every 3-4 d, a 5 mL aliquot from each flask was fixed in Lugol's iodine (final concentration = 2%) and cells

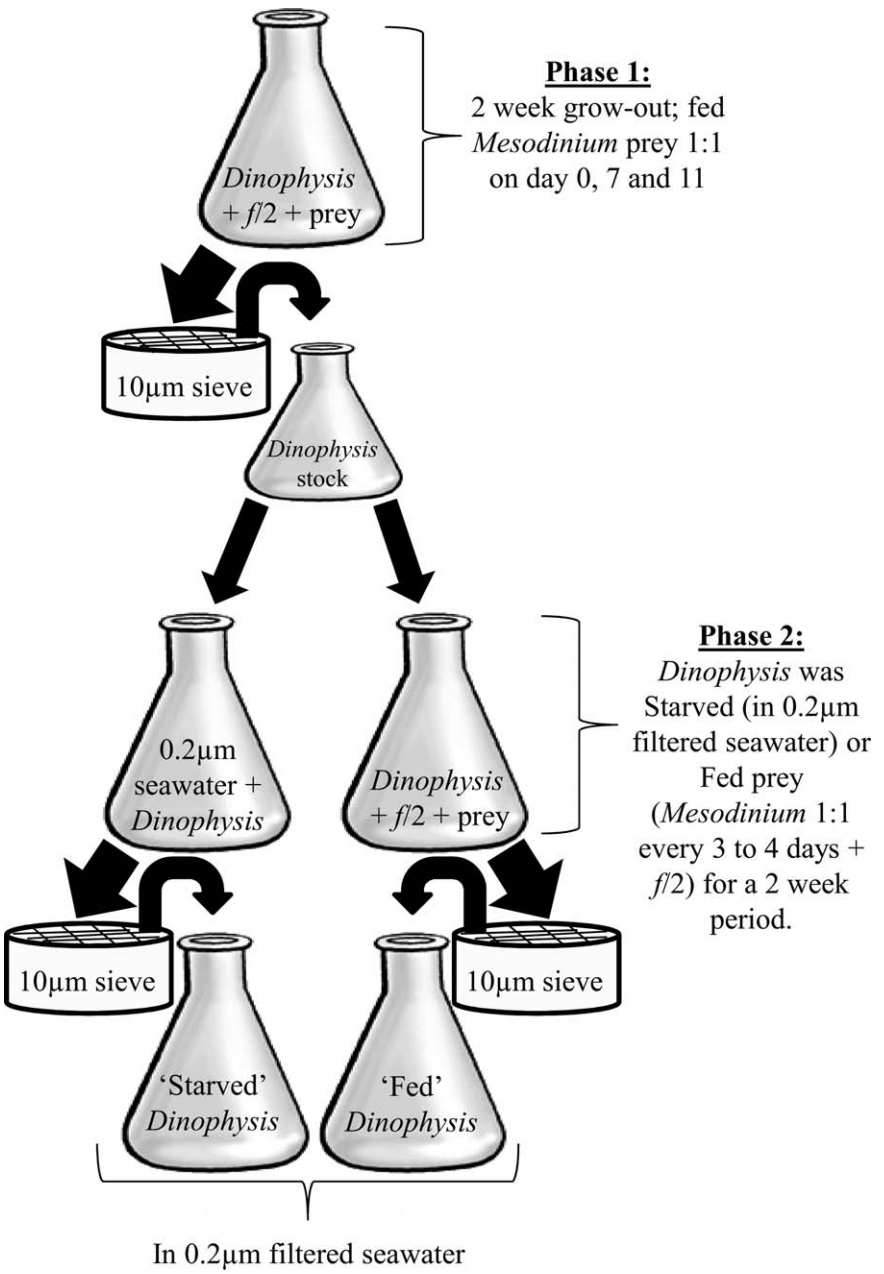

Fig. 1. Detailed description of Phase 1 and Phase 2 preconditioning of *Dinophysis* cultures prior to nutrient and prey amendment experiments.

were enumerated with a 1 mL Sedgewick-Rafter chamber using a compound microscope.

Growth rates were calculated during the exponential growth phase (persisting 4-8 d depending on food source ratio) using the formula: $\mu = \ln(B_t/B_0)/t$, where B_0 and B_t are the initial and final cell densities and t is the incubation duration in days. Differences in growth rates among treatments were evaluated with a One Way ANOVA with post hoc Tukey multiple comparisons tests using Sigma Stat software embedded within Sigma Plot 11.0. Additionally, growth rates of *D. acuminata* over the varying *M. rubrum* ratios were fitted using linear regression as well as a Michaelis–Menten model using curve fitting functions within Kaleidagraph (Synergy Software; version 4.5) and μmax (maximum growth rate) and K_s (half saturation constant, as prey concentration) were derived.

Uptake rates of nitrogenous nutrients by starved and fed *Dinophysis* cultures

^{15}N tracer experiments were conducted using both starved and fed *D. acuminata* cultures (*see* below) to quantify uptake rates of nitrogenous compounds by starved and fed

Table 1. Nutrient concentrations (μmol L^{-1}) in experimental controls (stock cultures). Experiments labeled same as in corresponding figures and tables. Data was not available for the time series experiment. DON, dissolved organic nitrogen. Data are means (SD).

		Nutrients in (μmol L^{-1})		
		Nitrate	**Ammonium**	**DON**
Short term	Starved	1.2 (0.3)	0.5 (0.3)	7.1 (0.4)
	Fed	2.5 (0.5)	1.3 (0.8)	8.9 (1.2)
M. rubrum 2:1	Without prey	0.7 (0.1)	2.0 (0.2)	n/a
	With prey	0.5 (0.1)	3.2 (0.3)	n/a
M. rubrum 10:1	Without prey	0.7 (0.1)	3.8 (0.7)	14.0 (1.9)
	With prey	0.3 (0.03)	2.1 (0.3)	16.7 (0.9)

Dinophysis. Uptake of ammonium, nitrate, and urea were measured using tracer additions (200 nmol L^{-1}, 200 nmol L^{-1}, and 50 nmol L^{-1}, respectively) of highly enriched (98–99%) ^{15}N-labeled compounds added as 180 and 4%, 70 and 140%, and 50 and 60% of ambient pools for starved and fed cultures, respectively. Immediately, prior to the start of the experiments both starved and fed cultures were sieved using a 10 μm mesh filter and washed into freshly made autoclaved, 0.2 μm-filtered aged seawater to ensure both cultures had the same background nutrient concentrations and minimal bacterial densities. Cultures were then distributed to sterile, triplicate 50 mL polystyrene flasks and incubated for 1 h at the conditions mentioned above. Turnover rates of nutrients were assumed to be minimal given the relatively short incubation period (Glibert et al. 1982) as were contributions of bacteria to the ^{15}N signal given their relatively low biomass (in cultures incubated over 6 d, *see* Results) and our use of GF/F glass fiber filters retain a smaller fraction of bacteria. At the end of the incubation, treatments in addition to control samples (no tracer added) were filtered onto pre-combusted (2 h at 450°C) 25mm GF/F glass fiber filters and frozen (−20°C). Samples were then dried at 60°C and pelleted for particulate nitrogen (PN) and ^{15}N analysis using a ThermoFinnigan Delta Plus XP isotope ratio mass spectrometer at the Kudela Lab in UC Santa Cruz. Uptake rates were calculated according to the mixing model of Montoya et al. (1996) using equations from (Orcutt et al. 2001). Differences in PN specific- and cell specific-uptake rates among treatments were elucidated using two-way ANOVAs with post hoc Tukey multiple comparisons tests where preconditioning (starved or fed) and nutrient source were the main effects using Sigma Stat software embedded within Sigma Plot 11.0.

Growth of cultures with multiple nutrient sources and prey densities

Experiments were conducted to isolate the effects of nutrients and prey on *D. acuminata* growth. Prior to the initiation of the experiments a 1-month, two-phase, preconditioning period was implemented (Fig. 1). For both phases, *M. rubrum* was added at $\sim$ 1 : 1 ratio to reflect prey densities in the field where *Dinophysis* spp. is typically food-limited (Kim et al. 2008; Riisgaard and Hansen 2009). The first phase was a grow-out period (2 weeks) in which *D. acuminata* was grown in nutrient replete medium (*f*/2 -Si) and fed *M. rubrum* at a ratio of $\sim$ 1 : 1 three times (day 0, day 7 and day 11; Fig. 1). After the 2 week period the culture was sieved through a 10 μm mesh (to rid the culture of residual *M. rubrum* and nutrients) and subsequently washed into autoclaved, 0.2 μm-filtered, aged seawater. These *D. acuminata* cells were then used to initiate phase two of the preconditioning period when they were starved or fed for another 2 weeks (Fig. 1). The starved *D. acuminata* culture was maintained in autoclaved, 0.2 μm-filtered, aged seawater. The fed culture was maintained in nutrient replete medium (*f*/2 -Si, made from autoclaved, 0.2 μm-filtered, aged seawater) and fed *M. rubrum* at a $\sim$ 1 : 1 ratio four times during the 2 week period (every 3-4 d). Given the 2 week period without prey, starved cultures were assumed to be relying primarily on photosynthesis while fed cultures were considered mixotrophic. Upon the end of phase two, the starved and fed *D. acuminata* cultures were sieved through a 10 μm mesh and were added into autoclaved, 0.2 μm-filtered, aged seawater to rid the fed culture of residual *M. rubrum* and nutrients and ensure cultures used for experiments had identical background nutrient levels (Table 1).

Preconditioned cultures (starved and fed) were used for experiments exploring the effects of different nutrients on the growth of *D. acuminata* (Table 2). For each of the two conditions (starved and fed), a control (Table 1) was established in addition to the following treatments: ammonium (50 μmol L^{-1}), nitrate (50 μmol L^{-1}), glutamine (25 μmol L^{-1} = 50 μmol L^{-1} N), vitamin B_{12} (100 pmol L^{-1}) and high molecular weight sewage treatment plant effluent (HMW STP; 50 μmol L^{-1} N), treatments matching and thus complementing field experiments conducted in Hattenrath-Lehmann et al. (2015). All treatment concentrations were within the range of total dissolved nitrogen levels found in Long Island estuaries (Gobler et al. 2004; Koch et al. 2012; Hattenrath-Lehmann et al. 2015), with the exception of glutamine which is typically present at submicromolar concentrations (Gobler et al. 2012) but was added at equimolar levels of N for comparison with other nitrogen sources. High molecular weight (> 1 kDa, Millipore) organic matter from sewage treatment plant effluent was isolated and concentrated from the Riverhead Sewer District plant which is located in Riverhead, New York, $\sim$ 1 km west of Meetinghouse Creek (Hattenrath-Lehmann et al. 2015). High molecular weight organic matter was isolated via tangential flow filtration as described by Gobler and Sanudo-Wilhelmy (2003). The use of tangential flow filtration ensures that high molecular weight organic material is concentrated but

Table 2. Summary of experimental setups for the growth of *Dinophysis* cultures with multiple nutrient sources and prey densities: Experiments #1, 2 and 3.

	Experiment #1	Experiment #2	Experiment #3
Preconditioning	Starved and fed	Starved and fed	Fed
Vessel	Six well plates	50 mL Nunc flasks	Six well plates
Incubation time	12 d	6 d	6 d
Sampling points	Every 4 d	Day 1 ($t = 0$), Day 6 ($t = f$)	Day 1 ($t = 0$), Day 6 ($t = f$)
Nutrient treatments	Control, ammonium, nitrate, glutamine, vitamin B_{12}, HMW STP		
Nutrient treatment dose	Single dose of 50 μmol L^{-1} N 100 pmol L^{-1} B_{12}	Two doses (day 0 and 3) of 25 μmol L^{-1} N 50 pmol L^{-1} B_{12}	Two doses (day 0 and 3) of 25 μmol L^{-1} N 50 pmol L^{-1} B_{12}
Mesodinium treatments	None	None	2:1 and 10:1 ratios

inorganic nutrient concentrations remained unchanged (Gobler and Sanudo-Wilhelmy 2003). The first experiment (Experiment #1; time series) was performed using six-well culture plates (10 mL per well) with a single dose of nutrients and aliquots removed every 4 d for cell enumeration over a 12 day period (Table 2). A second experiment (Experiment #2) was shorter term (6 d) and complementary to the first experiment (used 50 mL polystyrene flasks, Thermo Scientific™) but was performed with nutrients added in two equal doses (day 0 and day 3) to match the above mentioned total concentrations (50 μmol L^{-1} N) but to avoid potential toxic effects of high ammonium (Collos and Harrison 2014) seen in initial experiments (Table 2). A third experiment (Experiment #3) was performed where the addition of *M. rubrum* (at ratios of 2 : 1 and 10 : 1) was used to assess the effects of nutrients on *D. acuminata* when *M. rubrum* is present and absent. This experiment was conducted for 6 d in six-well culture plates, nutrients were added in two equal doses as above and used *Dinophysis* cells preconditioned as "fed" (Table 2). At the end of experiments, aliquots were preserved in Lugol's iodine and cell densities were enumerated as above. Differences in cell densities among treatments were elucidated using two-way ANOVAs with post hoc Tukey multiple comparisons tests where either pre-conditioning (starved or fed), or food level (with or without *M. rubrum*) and nutrient source were the main effects using Sigma Stat software embedded within Sigma Plot 11.0. Additionally, for the third set of experiments, the N contribution of nutrients and *M. rubrum* (at ratios of 2 : 1 and 10 : 1) to *Dinophysis* growth was calculated by converting *Dinophysis* growth ($t_{final} - t_{initial}$ abundances) and abundances of *M. rubrum* added to experimental units (assuming negligible growth) to N equivalents. This was done by using previously published carbon contents of both species (Riisgaard and Hansen 2009) and converting those to N based on Redfield ratios, resulting in N contents of 158 pg $cell^{-1}$ and 63 pg $cell^{-1}$, for *D. acuminata* and *M. rubrum*, respectively. The contribution of nutrients to *Dinophysis* growth was considered the difference between *Dinophysis* growth and the calculated contribution of *M. rubrum*.

Bacterial experiments

The effects of nutrients and *D. acuminata* on the heterotrophic bacterial community within cultures were examined to assess bacterivory in *D. acuminata*. To remove *D. acuminata* without changing bacterial densities, a portion of the well mixed stock flask pre-conditioned as fed (as above) was filtered through a 5 μm polycarbonate filter. Six-well plates with and without *D. acuminata* were then established in parallel to assess differences in the growth of the heterotrophic bacterial community. A no nutrient control was established and nutrients (HMW STP and glutamine) that elicited an increase in *D. acuminata* densities in the previous experiments were added and run in parallel with the short term experiment described in the above nutrient experiments section. At the start and end of experiments, whole water samples were preserved in 10% buffered formalin (0.5% v/v final), stored at −80°C, and analyzed flow cytometrically to quantify the abundance of heterotrophic bacteria. Samples were stained with SYBR Green I and heterotrophic bacteria were quantified using a FACScan (BD®) flow cytometer (Jochem 2001). To determine the potential contribution of bacterivory to the growth of *D. acuminata*, abundances of both populations were converted to C equivalents using previously published carbon contents of 895 pg $cell^{-1}$ (Riisgaard and Hansen 2009) and 20 fg $cell^{-1}$ (Fukuda et al. 1998; Ducklow 2000), for *D. acuminata* and heterotrophic bacteria, respectively.

Nutrient analyses

To determine ambient nutrient concentrations in stock cultures (Table 1), filtrate was made using precombusted (4 h at 450°C) glass fiber filters (GF/F, 0.7 μm pore size) and frozen in acid washed scintillation vials. Filtrate was analyzed colorimetrically for nitrate, ammonium, and urea (Jones 1984; Parsons et al. 1984) using a spectrophotometeric microplate reader.

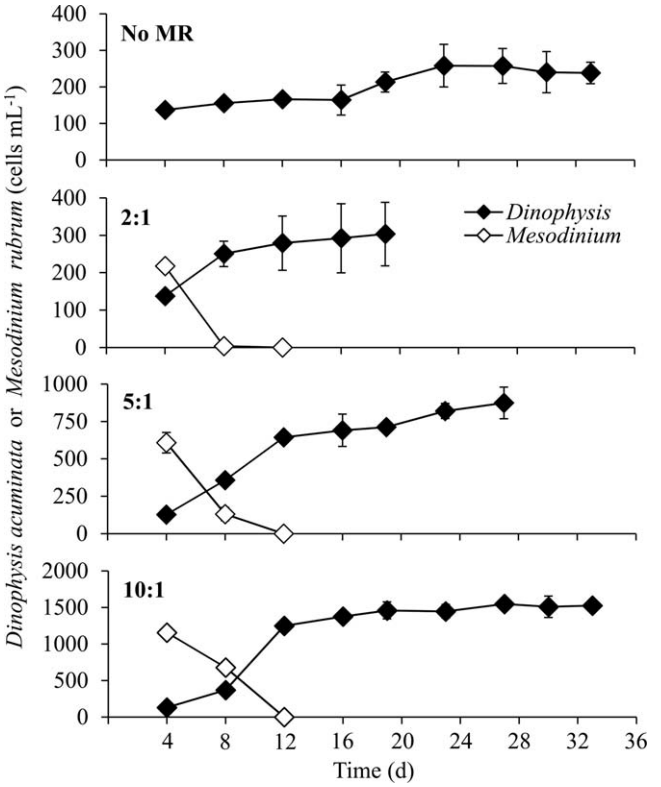

Fig. 2. Abundance of *D. acuminata* (cells mL^{-1}) in cultures fed varying *M. rubrum* ratios (no MR, 2 : 1, 5 : 1 and 10 : 1). Points are means while error bars represent standard deviation of triplicate measurements.

Results

Growth of *Dinophysis acuminata* under varying *Mesodinium rubrum* ratios

The growth rates of *D. acuminata* cultures varied over the range of *M. rubrum* : *D. acuminata* ratios (Fig. 2; Table 3). *D. acuminata* growth rates were low (0.06 ± 0.01 d^{-1}) in the absence of prey reaching peak densities of 258 ± 58 cells mL^{-1}. With increasing prey ratios (2 : 1, 5 : 1, and 10 : 1) the growth rates of *D. acuminata* increased significantly (0.15 ± 0.04, 0.20 ± 0.01, 0.28 ± 0.01 d^{-1}; $p < 0.01$, Tukey) with maximal *D. acuminata* densities of 303 ± 85, 873 ± 106, and 1549 ± 65 cells mL^{-1}, respectively (Fig. 2; Table 3). For all prey ratios, *M. rubrum* was consumed by *D. acuminata* within the first 8 d of the experiment (Fig. 2). *M. rubrum* growth in control cultures without *D. acuminata* was negligible, ranging from 0 d^{-1} to 0.04 d^{-1}. *D. acuminata* growth rates as a function of prey : predator ratio (Table 3) were fit better to a linear function ($R^2 = 0.91$) than a Michaelis–Menten equation ($R^2 = 0.79$), which had a maximum growth rate of 0.36 ± 0.07 d^{-1} and a prey : predator ratio of 3.23 ± 1.61 (323 ± 161 cells mL^{-1} *M. rubrum*) sustaining half the maximum growth rate (K_s). This was likely because growth saturation was not reached under the prey densities used as evidenced by the calculated maximum growth rate which was not reached during these experiments. This calculated maximal growth rate, however, matched the highest experimental growth rates of 0.36 ± 0.01 d^{-1} observed in glutamine grown cultures with a 10 : 1 feeding ratio (Table 4).

Table 3. *D. acuminata* growth rates (d^{-1}) as a function of *M. rubrum* feeding ratios. Data are means of triplicate measurements (SD). Letters indicate Tukey multiple comparisons results ($p < 0.05$).

Mesodinium feeding ratio	*D. acuminata* growth rate (μ d^{-1})
0	0.06 (0.01)[a]
2:1	0.15 (0.04)[b]
5:1	0.20 (0.01)[b]
10:1	0.28 (0.01)[c]

Uptake of various N sources by starved and fed cultures of *Dinophysis acuminata*

Pre-conditioning (starved vs. fed) and nutrients significantly affected both PN specific- and cell specific-uptake rates of *D. acuminata* and there was a significant interaction between these factors as the response to preconditioning differed among the different nutrients investigated ($p < 0.001$ for all, two-way ANOVA on uptake rates; Fig. 3; Table 5). Ammonium uptake rates were significantly higher ($p < 0.001$, Tukey) than those of any other nitrogen source followed by urea and nitrate (Fig. 3; Table 5). Ammonium and urea uptake rates were both significantly higher ($p < 0.01$, Tukey) in fed *D. acuminata* cultures compared to starved cultures, whereas the opposite was observed for nitrate uptake rates (Fig. 3; Table 5). PN specific- and cell specific-uptake rates followed a similar pattern with the exception that there was no significant difference in cell specific-urea uptake rates between fed and starved cultures of *D. acuminata* (Fig. 3; Table 5).

The effects of nutrients on starved and fed *Dinophysis acuminata*

Time series experiment

Pre-conditioning (starved vs. fed) and nutrients significantly affected *D. acuminata* densities and there was a significant interaction between these factors as the response to preconditioning differed among the different nutrients investigated ($p < 0.001$ for all, two-way ANOVA on final densities; Fig. 4). In starved cultures, the addition of HMW STP significantly ($p < 0.01$, Tukey) increased cell densities by 25% compared to the control while all other nutrient additions slightly decreased *D. acuminata* densities (by 2-13%; Fig. 4). In the fed cultures, however, HMW STP, glutamine, and ammonium additions increased *D. acuminata* densities by 64, 24, and 13%, respectively, compared to the control

Table 4. Cellular division rates (calculated using initial and final cell densities) of *D. acuminata* from experiments comparing the effects of varying *M. rubrum* prey : predator ratios and nutrients. Data are means (SD). Asterisks indicate growth rates with *M. rubrum* added that were significantly different from their without prey counterpart ($p < 0.05$, Tukey) whereas those in bold indicate treatments that are significantly different from their respective control.

	Mean cellular division rates (μ d^{-1})			
	M. rubrum 2 : 1		*M. rubrum* 10 : 1	
	Without prey	With prey	Without prey	With prey
Control	0.05 (0.02)	0.10 (0.01)*	0.07 (0.01)	0.31 (0.01)*
B12	0.05 (0.01)	0.10 (0.01)*	0.08 (0.02)	0.30 (0.01)*
HMW STP	**0.12 (0.01)**	**0.17 (0.02)***	**0.16 (0.01)**	0.33 (0.002)*
Nitrate	0.05 (0.02)	0.11 (0.01)*	0.07 (0.01)	**0.34 (0.002)***
Ammonium	0.07 (0.01)	**0.17 (0.01)***	**0.14 (0.02)**	**0.34 (0.004)***
Glutamine	0.04 (0.03)	0.12 (0.02)*	**0.13 (0.01)**	**0.36 (0.01)***

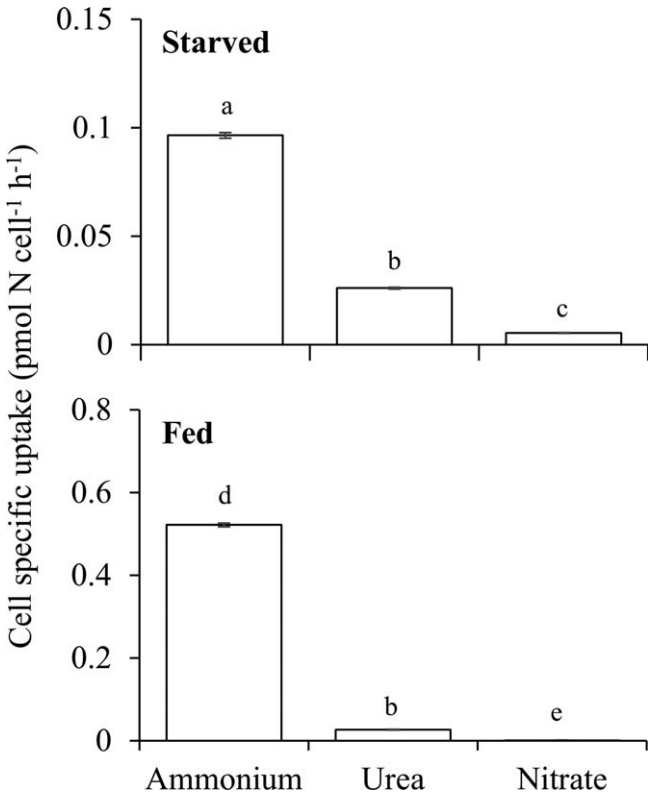

Fig. 3. Cell specific uptake (pmol N $cell^{-1}$ h^{-1}) of various ^{15}N labeled nitrogen sources (ammonium, urea, nitrate) in *D. acuminata* cultures that were preconditioned as starved or fed. Bars are means while error bars represent standard deviation of triplicate measurements. Letters indicate Tukey multiple comparisons results ($p < 0.05$).

($p < 0.01$; Tukey) while changes due to the addition of nitrate and B_{12} were negligible (Fig. 4).

Short term experiment

Pre-conditioning (starved vs. fed) and nutrients significantly affected *D. acuminata* densities and there was a significant interaction between these factors as the response to preconditioning differed among the different nutrients investigated ($p < 0.001$ for all, two-way ANOVA; Fig. 5). *D. acuminata* densities in control fed cultures increased by 200 cells mL^{-1} while growth in control starved cultures was negligible (Fig. 5). Compared to control cultures, the addition of HMW STP and glutamine significantly increased *D. acuminata* densities by 120% and 150% for starved, and 97% and 62% for fed cultures, respectively ($p < 0.001$, Tukey; Fig. 5). The addition of ammonium significantly decreased (77%) *D. acuminata* densities in starved cultures, but significantly increased (60%) densities in fed cultures ($p < 0.001$, Tukey; Fig. 5). Similar to the previous experiment, the addition of B_{12} and nitrate had little effect on *D. acuminata* densities (Fig. 5).

Table 5. PN and cell specific uptake of various ^{15}N labeled nitrogen sources (ammonium, urea, nitrate) in *D. acuminata* cultures that were preconditioned as starved or fed. Data are means (SD) of triplicate flasks. Letters indicate Tukey multiple comparisons results ($p < 0.05$).

		PN specific uptake (h^{-1} × 10^{-3})	Cell specific uptake (pmol N $cell^{-1}$ h^{-1})
Starved	Ammonium	8.4 (0.1)[a]	0.096 (0.001)[a]
	Urea	2.2 (0.1)[b]	0.026 (0.0003)[b]
	Nitrate	0.43 (0.01)[c]	0.005 (0.0001)[c]
Fed	Ammonium	46.9 (0.6)[d]	0.52 (0.004)[d]
	Urea	2.5 (0.1)[e]	0.027 (0.001)[b]
	Nitrate	0.12 (0.03)[f]	0.001 (0.0003)[e]

The effects of varying ratios of *Mesodinium rubrum* and nutrients on *Dinophysis acuminata*

Prey availability (with vs. without *M. rubrum*) and nutrients significantly affected *D. acuminata* growth at both

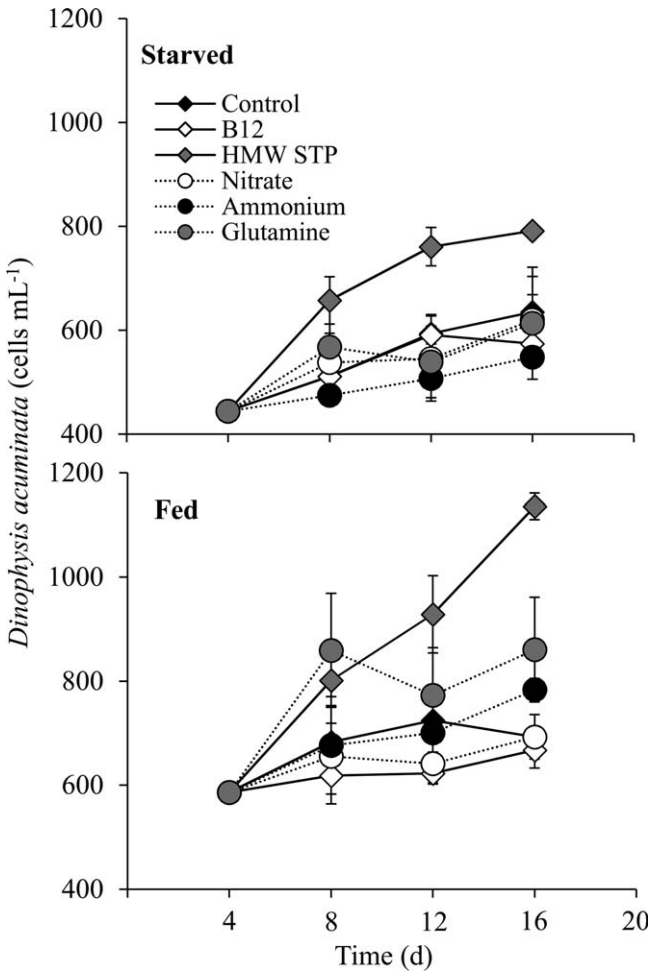

Fig. 4. Experiment #1, time series: *D. acuminata* densities (cells mL^{-1}) in cultures that were preconditioned as starved (not offered *M. rubrum* or nutrients) or fed (offered *M. rubrum* and nutrients) and then offered various nutrients in the absence of *M. rubrum* and incubated over a 12 day period. Points are means while error bars represent standard deviation of triplicate measurements. HMW STP, high molecular weight sewage treatment plant effluent.

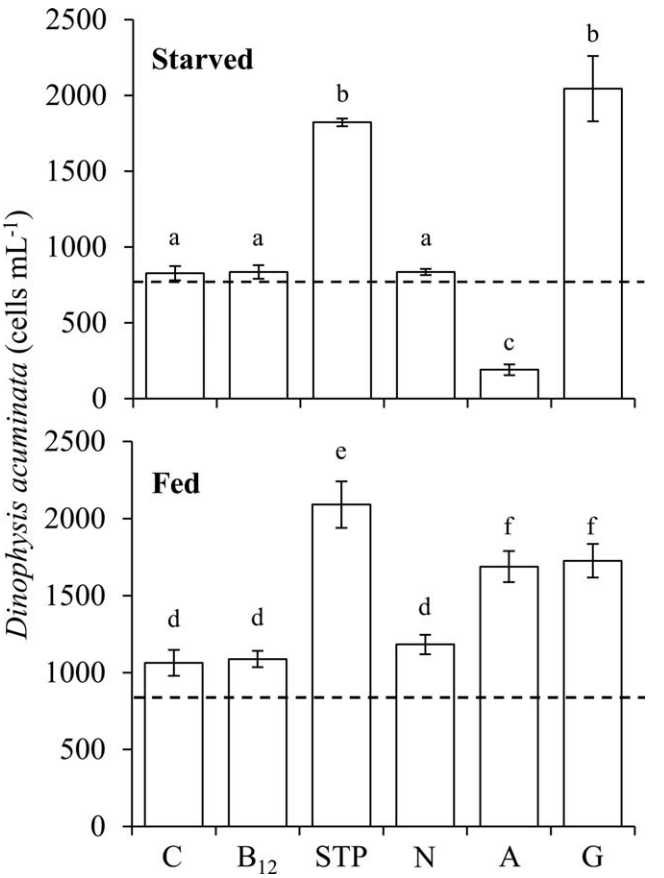

Fig. 5. Experiment #2, Short term experiment: Final cell yields of *D. acuminata* (cells mL^{-1}) in cultures that were preconditioned as starved (not offered *M. rubrum* or nutrients) or fed (offered *M. rubrum* and nutrients) and then offered various nutrients in the absence of *M. rubrum* over a 6 d incubation. Bars are means while error bars represent standard deviation of triplicate measurements. The dotted line denotes starting densities for both cultures. Letters indicate Tukey multiple comparisons results ($p < 0.05$). C, Control; B12, vitamin B12; STP, high molecular weight sewage treatment plant effluent; N, nitrate; A, ammonium; and G, glutamine.

2 : 1 and 10 : 1 feeding ratios and there was a significant interaction between these factors as the response to nutrients differed among the different prey levels investigated ($p < 0.001$ for all, two-way ANOVA; Fig. 6). Compared to initial *D. acuminata* concentrations, control cultures without *M. rubrum* grew 1.3 to 1.5-fold, while control cultures fed *M. rubrum* at 2 : 1 and 10 : 1 ratios grew 1.8 and 6.3-fold, respectively (Fig. 6). Across all nutrient treatments, the addition of *M. rubrum* significantly ($p < 0.001$, Tukey) increased *D. acuminata* densities 1.4 to 1.9-fold at a 2 : 1 feeding ratio, and 2.8 to 4.9-fold at a 10 : 1 ratio, compared to their respective no *M. rubrum* treatments (Fig. 6). Densities of *D. acuminata* cultures fed a 2 : 1 prey ratio significantly increased with the addition of HMW STP (53%) and ammonium (55%), while their no *M. rubrum* counterparts only significantly increased with the addition of HMW STP (53%; $p < 0.001$ for all, Tukey; Fig. 6), compared to their respective controls. When *M. rubrum* was fed to cultures at ratios of 10 : 1, *D. acuminata* densities increased with the addition of HMW STP (16%), nitrate (20%), ammonium (23%), and glutamine (40%) compared to the control ($p < 0.001$, Tukey), while densities of cultures without *M. rubrum* significantly increased with the addition of HMW STP (76%), ammonium (50%) and glutamine (44%; $p < 0.01$, Tukey; Fig. 6). Consistent with other experiments the addition of B_{12} had no effect on *D. acuminata*. While the effects of nutrients on *D. acuminata* were less pronounced at higher *M. rubrum* densities (10 : 1) compared to those grown on lower densities or without *M. rubrum* (Fig. 6), nutrients significantly enhanced the growth of *D. acuminata* cultures at all prey levels; the highest

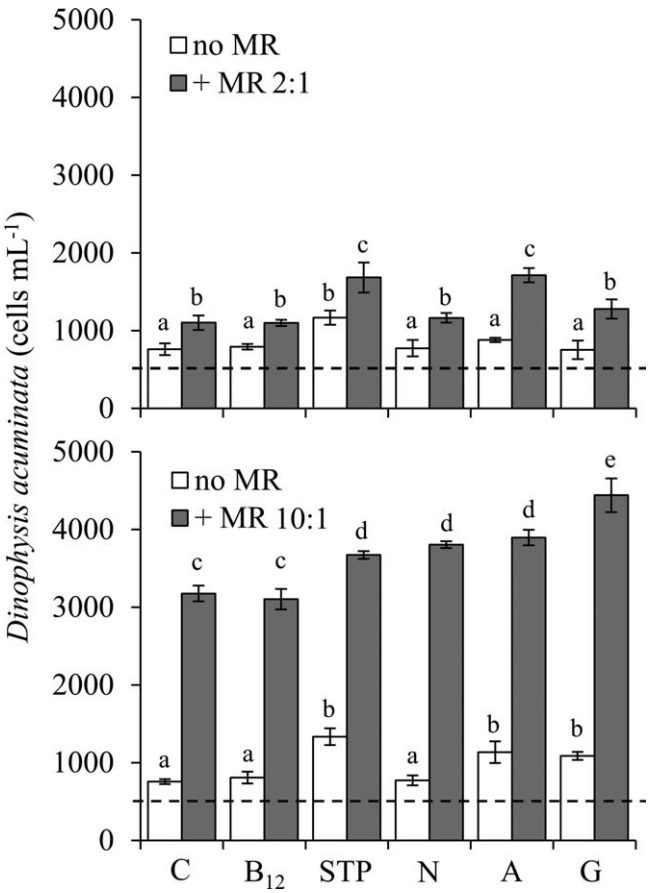

Fig. 6. Experiment #3, prey (presence vs. absence) and nutrients: Final cell yields of *D. acuminata* (cells mL^{-1}; preconditioned as fed) from experiments comparing the effects of the presence vs. absence of *M. rubrum* at varying feeding ratios (2 : 1 and 10 : 1) and the addition of nutrients on *D. acuminata* cultures. Bars are means while error bars represent standard deviation of triplicate measurements. The dotted line denotes starting densities for both experiments. Letters indicate Tukey multiple comparisons ($p < 0.05$). Treatments as in Fig. 4.

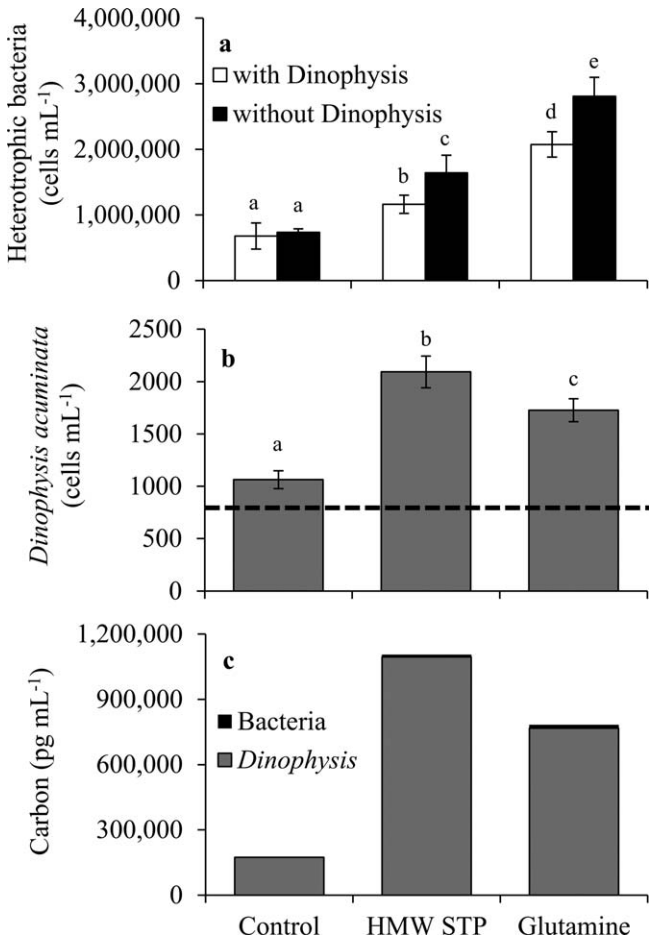

Fig. 7. (A) The effects of nutrients and the presence of *D. acuminata* on heterotrophic bacterial densities (cells mL^{-1}). (B) The effects of nutrients on *D. acuminata* densities (cell mL^{-1}). The dotted line denotes starting densities. (C) Carbon-specific biomass (pg C mL^{-1}) of the reduction in heterotrophic bacteria in the presence of *Dinophysis* and increase in *Dinophysis* during experiments. Bars are means while error bars represent standard deviation. Letters indicate Tukey multiple comparisons results ($p < 0.05$).

D. acuminata densities were achieved in cultures with high *M. rubrum* densities and nutrients added.

The effects of nutrients and *D. acuminata* on bacterial densities

Both nutrients and the presence of *D. acuminata* (with vs. without) significantly ($p < 0.01$, two-way ANOVA) affected heterotrophic bacterial densities in fed cultures of *D. acuminata*, but there was no interaction between these factors (Fig. 7A). In cultures with *D. acuminata*, the addition of HMW STP and glutamine significantly ($p < 0.05$, Tukey) increased heterotrophic bacterial densities 71% and 205%, respectively, whereas in cultures without *D. acuminata* bacterial densities increased 120% and 280%, respectively (Fig. 7A). Heterotrophic bacteria densities in cultures without *Dinophysis* were 8-41% higher than cultures with *Dinophysis* (Fig. 7A), suggesting that *Dinophysis* had an inhibitory effect (i.e., grazing, allelopathy, nutrient competition) on bacteria. Contrary to patterns in heterotrophic bacterial abundances, HMW STP and glutamine significantly ($p < 0.001$, Tukey) increased *D. acuminata* densities 97% and 62%, respectively, compared to the control (Fig. 7B) representing increases in biomass of 10.9 ± 1.3, and $7.6 \pm 0.97 \times 10^5$ pg C mL^{-1}, respectively (Fig. 7C). The differences in heterotrophic bacterial carbon concentrations in treatments with and without *D. acuminata* were two to three orders of magnitude lower than these C specific increases in *D. acuminata* (Fig. 7C). As such, decreases in heterotrophic bacterial carbon due to the presence of *D. acuminata* represented less than 2% of the carbon increase in *Dinophysis* and thus did not contribute appreciably to increases in *D. acuminata* biomass during experiments.

Discussion

While recent evidence (Hattenrath-Lehmann et al. 2015) indicated that blooms of the mixotrophic dinoflagellate, *D. acuminata*, could be stimulated by N loading, it is still unclear whether these effects were direct (via nutrient uptake by *Dinophysis*) or indirect (via nutrient stimulation of prey). During this study, ^{15}N tracer experiments demonstrated that *D. acuminata* assimilates ammonium and urea, but not nitrate, at high rates. To our knowledge, this study is the first example of a free-living kleptoplastidic flagellate assimilating exogenous dissolved nutrients. High molecular weight sewage treatment plant effluent and glutamine significantly and consistently enhanced the growth of *D. acuminata* in both starved and fed cultures compared to respective controls, while ammonium did so only when cultures were preconditioned as fed, and nitrate significantly increased densities of fed cultures only when *D. acuminata* was offered *M. rubrum* ratios of 10 : 1. In contrast, the addition of vitamin B_{12} had no effect on *D. acuminata* densities in any experiment. Across all experiments, growth rates of fed cultures were significantly higher than those of their starved counterparts. While the effects of nutrients on *D. acuminata* were smaller at higher *M. rubrum* densities (10 : 1) compared to those grown with lower densities or without *M. rubrum*, nutrients consistently and significantly enhanced the growth of both starved and fed cultures in all experiments. Heterotrophic bacterial biomass could not account for the growth of *Dinophysis*, indicating that nutrients and not bacterivory supported *Dinophysis* growth in cultures where *M. rubrum* was not added. Collectively, this study unequivocally demonstrates that the assimilation of nitrogenous nutrients directly promote the growth of *Dinophysis*.

Growth rates of *Dinophysis acuminata* compared to other cultures

Growth rates of *Dinophysis* spp. cultures have been found to range from 0.06 d^{-1} to 0.95 d^{-1} and have been shown to vary as a function of temperature, prey concentration, and light (Park et al. 2006; Kim et al. 2008; Kamiyama and Suzuki 2009; Riisgaard and Hansen 2009; Tong et al. 2010; Nagai et al. 2011; Nielsen et al. 2012, 2013). Our growth rate experiments conducted at 18°C with a *D. acuminata* culture established from New York yielded cellular division rates (no prey) of $\sim$ 0.1 d^{-1} and cellular division rates up to 0.36 d^{-1} when cultures were fed *M. rubrum* at a 10 : 1 ratio with the addition of glutamine. It is possible, however, that growth rates are underestimates due to the 3-4 d gap between sampling points that may have missed periods of maximal rates. Grow rates were, however, within the range of those observed for another North American strain of *D. acuminata* cultured using a cryptophyte strain, *Geminigera cryophila*, and *M. rubrum* isolated from the Ross Sea (0.11 d^{-1} and 0.23 d^{-1} at 4°C and 10°C, respectively; Tong et al. 2010). *D. acuminata* cultured using the same *M. rubrum* and *T. amphioxeia* cultures as the present study yielded growth rates of 0.13–0.51 d^{-1} (Nielsen et al. 2012). While comparisons of growth rates between studies is difficult given disparate culturing conditions, overall the growth rate of the New York strain of *D. acuminata* was within the range observed in other culture studies.

Dinophysis and nutrients

Investigations of the effects of nutrients on the growth of *Dinophysis* have been rare, contradictory, and often focus on field correlations and experiments that are unable to isolate potential indirect effects of nutrients on prey items (Delmas et al. 1992; Giacobbe et al. 1995; Koukaras and Nikolaidis 2004; Seeyave et al. 2009; Hattenrath-Lehmann et al. 2015). While some field studies have found no relationship between *Dinophysis* densities and nutrient concentrations (Delmas et al. 1992; Giacobbe et al. 1995; Koukaras and Nikolaidis 2004), Seeyave et al. (2009) demonstrated *Dinophysis* dominated-communities (91% of total biomass as C) have a high affinity for ammonium. Recent ecosystem-based studies in NY estuaries (Hattenrath-Lehmann et al. 2015) have demonstrated that *Dinophysis* cell division rates can be promoted by the accelerated delivery of both inorganic (nitrate, ammonium) and organic N (glutamine, HMW STP). The only culture studies investigating the effects of nutrients on *Dinophysis* growth concluded that both organic matter originating from sonicated *M. rubrum* cultures (Nagai et al. 2011) and dissolved inorganic nitrogen (Tong et al. 2013) had no effect on the growth of *D. acuminata* cultures. The present culture study, in agreement with (Hattenrath-Lehmann et al. 2015), demonstrated using multiple experimental approaches that both inorganic and organic nutrients significantly enhanced the growth of *D. acuminata*. More specifically, we were able to demonstrate that these nutrient effects were direct as enhancements were seen in cultures of *D. acuminata* where no *M. rubrum* was added and *D. acuminata* cultures were shown to assimilate ammonium at high rates.

During this study, *Dinophysis* cultures were capable of assimilating ammonium and urea, but not nitrate, at rapid rates, a finding consistent with growth experiments that found significant growth with ammonium, but not nitrate (urea not examined) and consistent with an ecosystem-based study of *Dinophysis*, that found ammonium uptake was highest followed by urea and nitrate (Seeyave et al. 2009). While our cultures contained bacteria, most bacteria likely passed through the filters used for ^{15}N measurements (Lee et al. 1995) and bacterial biomass was too low to appreciably contribute to uptake rates ($<$ 1% of *Dinophysis* C-based biomass; Fig. 7). Regarding ammonium, the difference in uptake between starved (lower) and fed (higher) cultures were consistent with growth experiments where ammonium enhanced the growth of fed cultures but suppressed growth in starved cultures. Moreover, ^{15}N-based ammonium uptake

rates for cultures pre-conditioned as starved (0.8 μmol L^{-1} d^{-1}) and fed (7.8 μmol L^{-1} d^{-1}) were within the range of those estimated for cultures grown on ammonium for multiple days (0.7-1.2 μmol L^{-1} d^{-1}) without prey but preconditioned as fed. Lower N uptake for cultures grown over multiple days could reflect an overestimate of N quotas using short term, daytime uptake rates given that diel variation in N uptakes are unknown. Further, cultures grown for multiple days without prey would exhaust their plastid reserves as they divide, effectively making them "starved," a condition that decreases their ability to use ammonium (Figs. 4, 5). Regarding nitrate, while uptake was low these values were different from zero ($p < 0.001$; *t*-test). This is the first study to measure direct nutrient assimilation by *Dinophysis*, a finding that supports the hypothesis that nutrient acquisition is a key aspect of *Dinophysis* ecophysiology. While additions of these tracers were, in some cases, above tracer levels (up to twice background levels), they were still submicromolar additions and thus far below levels typically observed during estuarine *Dinophysis* blooms (Hattenrath-Lehmann et al. 2015) and clearly demonstrate the ability of *Dinophysis* to assimilate nutrients directly. Further studies are needed to precisely quantify nutrient assimilation kinetics for this alga.

High molecular weight sewage treatment plant effluent and glutamine significantly and consistently enhanced the cellular division rates of *D. acuminata* cultures, a finding that replicates our prior ecosystem-based experiments (Hattenrath-Lehmann et al. 2015). While it is unclear whether nitrogen or carbon from these organic nutrients are stimulating *D. acuminata* growth, given that these enhancements were observed in starved cultures it suggests that *Dinophysis* is capable of osmotrophy (assimilation of organic compounds). While Nagai et al. (2011) did not observe any growth rate enhancements with the addition of organic substances, they did demonstrate that organic substances enhanced *Dinophysis* toxicity. A study by Graneli et al. (1997) focusing on the nutritional capabilities of *Dinophysis*, however, could not resolve whether dark C uptake by *Dinophysis* was due to phagotrophy or osmotrophy.

While ammonium significantly and consistently increased *Dinophysis* densities in experiments using field populations (Hattenrath-Lehmann et al. 2015), it did so in fed, but not starved, cultures of *D. acuminata*. When *Dinophysis* was starved, the addition of ammonium inhibited growth likely due to toxicity (Collos and Harrison 2014). We suggest that when *Dinophysis* is starved, the threshold of ammonium toxicity is lower compared to when it has been well fed, perhaps due to fewer functioning plastids and an overall compromised cell physiology. Additionally, observations (Fig. 6 bottom panel) suggest that *Dinophysis* densities are enhanced by ammonium when it is actively feeding on *M. rubrum* as well as when it has recently fed and therefore likely continues to grow on reserve nutrition (Reguera et al. 2012). Regardless, collective observations from this study and Hattenrath-Lehmann et al. (2015) demonstrate that ammonium stimulates *D. acuminata* cellular division rates when cells have recently fed and are, thus, likely physiologically healthy.

In contrast to other nutrients examined, nitrate significantly increased *D. acuminata* densities only when *D. acuminata* was also offered *M. rubrum* ratios of 10 : 1 and even under this circumstance, the enhanced growth was minor. This is consistent with field experiments in Hattenrath-Lehmann et al. (2015) where only one third of experiments showed a significant increase in *D. acuminata* densities with the addition of nitrate. Since nitrate only enhanced *D. acuminata* growth in the presence of high prey densities and since assimilation rates of ^{15}N labeled nitrate were negligible, we hypothesize that nitrate indirectly stimulated the growth of *D. acuminata* via uptake of nitrate by its prey, *M. rubrum*. Alternatively, given that in some dinoflagellates nitrate reduction reactions take place within the plastid (Fritz et al. 1996) and *Dinophysis* may have limited functional control of these plastids (Wisecaver and Hackett 2010; Hansen et al. 2013), perhaps they need to be actively feeding on- and acquiring plastids from- *M. rubrum* to utilize nitrate. Field observations and correlations, as well as nutrient uptake studies, demonstrated that *M. rubrum* is capable of using nitrate, ammonium and DON (Wilkerson and Grunseich 1990; Crawford et al. 1997; Herfort et al. 2012; Hansen et al. 2013). Myung et al. (2013) also demonstrated that a culture of *M. rubrum* was able to grow for $\sim$ 6 weeks in the absence of its cryptophyte prey, further evidencing its autotrophic capabilities and the role *M. rubrum* may play in the indirect stimulation of *D. acuminata* blooms. During this study, however, *M. rubrum* did not grow in control cultures that were nutrient replete and without *Dinophysis* or cryptophyte prey. This finding strongly suggests that increases in *Dinophysis* growth rates in the presence of prey and nutrients were direct effects (i.e., mixotrophy) rather than being due to an indirect effect (i.e., nutrient use by *M. rubrum* prey and subsequent consumption by *Dinophysis*).

Recent studies have suggested that *Dinophysis* populations can be food limited (i.e., *M. rubrum*) during bloom events (Kim et al. 2008; Riisgaard and Hansen 2009). Using data from experiment #3 (Fig. 6) we estimated the contribution of dissolved nutrients to *Dinophysis*' cellular N demand (*see* Methods) based on two assumptions: (1) negligible *M. rubrum* growth, and (2) constant C : N contents and cellular volumes (for predator and prey). These calculations indicated that the contribution of nitrogenous nutrients to *D. acuminata* increases with decreasing *M. rubrum* prey abundance (Fig. 6). Therefore, we suggest that in an ecosystem setting, *Dinophysis* may acquire equal or greater amounts of its cellular N requirement from nutrients (inorganic and organic). For example, according to our calculations cultures were found to obtain up to 60% of their cellular N from dissolved nutrients and cultures fed *M. rubrum* assimilated twice as much ammonium. This further illustrates that *D. acuminata*

is a true mixotroph that does not realize its full growth potential without both food and nutrients.

Mixotrophy has been shown to significantly increase the growth rates of several HABs in comparison to strict phototrophy (Jeong et al. 2005; Stoecker et al. 2006; Burkholder et al. 2008 and references therein; Kim et al. 2008). Consistent with these studies, we found that across all nutrient treatments, mixotrophic growth rates (with prey and nutrients) were significantly higher than those grown with nutrients or prey alone (Table 4). While the addition of nutrients increased the growth rates of cultures with and without prey compared to their respective controls, maximal growth rates were obtained when *Dinophysis* was fed *M. rubrum* at a 10 : 1 ratio in combination with the addition of glutamine (0.36 ± 0.01 d^{-1}; Table 4).

Dinophysis and bacterial associations

All *Dinophysis* culture investigations conducted thus far have maintained cultures under xenic conditions (i.e., with bacteria; Park et al. 2006; Kim et al. 2008; Hackett et al. 2009; Kamiyama and Suzuki 2009; Riisgaard and Hansen 2009; Tong et al. 2010; Fux et al. 2011; Nagai et al. 2011; Tong et al. 2011; Nielsen et al. 2012, 2013; Smith et al. 2012), and yet, to date no study has assessed the relationship between bacteria and *Dinophysis*. In this study, heterotrophic bacterial abundances were lower in the presence of *D. acuminata* compared to parallel treatments without *D. acuminata*, suggesting this alga inhibited bacterial growth via nutrient competition, allelopathy, or bacterivory, with the later creating the prospect that nutrient effects on *D. acuminata* may be indirect. There were multiple lines of evidence that demonstrate this was not the case, however. *D. acuminata* growth was highest with the addition of HMW STP effluent while the decrease in bacterial abundances was higher in the glutamine treatment. Additionally, reductions in heterotrophic bacterial biomass in the presence of *D. acuminata* accounted for $< 2\%$ of the observed growth by *Dinophysis* in treatments. Finally, while bacteria are well known for their ability to liberate ammonium from organic N compounds (Kirchman 2008) and the growth of *D. acuminata* was promoted by organic N compounds, the growth response to organic N was often larger than the response from equimolar amounts of ammonium. And, *D. acuminata* growth was also promoted by inorganic N and *D. acuminata* growth responses to organic N were higher than would have been predicted even if all of the organic nitrogen had been remineralized to ammonium by bacteria. Therefore, bacterial remineralization of organic N could not quantitatively account for the growth response of *D. acuminata* during experiments. Collectively, this suggests that nutrient assimilation supported the growth by *Dinophysis* in cultures where *M. rubrum* was not added. While bacterivory could explain the changes seen in heterotrophic bacteria abundances when *D. acuminata* was present, other processes such as an allelopathic effect of *Dinophysis* on bacteria, attachment of heterotrophic bacteria to *Dinophysis*, and competition for nutrients could also have reduced bacterial levels when grown in the presence of *D. acuminata*.

While some *Dinophysis* spp. are known to harbor endosymbiotic bacteria (Lucas and Vesk 1990), their interactions with free-living heterotrophic bacteria are poorly understood (Berland et al. 1995; Reguera et al. 2012). In this study, the inability to culture *Dinophysis* with the use of antibiotics could be due to a close association between *Dinophysis* and bacteria. Moreover, a recent study demonstrated that vitamins (B_1 and B_{12}) significantly enhanced *D. acuminata* densities in bloom water from two different estuaries (Hattenrath-Lehmann et al. 2015), providing a potential link to the *Dinophysis*-bacteria relationship given that bacteria are B-vitamin producers (Raux et al. 2000). A series of recent studies have demonstrated that the growth of phytoplankton can be strongly dependent on vitamin B_{12} production by bacteria (Croft et al. 2005; Kazamia et al. 2012). Further, ecosystem-based studies have shown that HABs can be promoted by high levels of B-vitamins (Koch et al. 2013) and recent culture studies have demonstrated that 91% of dinoflagellates surveyed ($n = 45$) required an exogenous source of vitamin B_{12} (Tang et al. 2010). This study, however, found that *D. acuminata* cultures were not affected by the addition of vitamin B_{12} with or without *M. rubrum* present. This may be due, in part, to bacterial production of B_{12} in these non-axenic cultures (Raux et al. 2000). In an ecosystem setting, where *Dinophysis*, *Mesodinium* and cryptophytes co-occur, an indirect effect of B_{12} on *Dinophysis* cannot be excluded given that Tang et al. (2010) found that other cryptophytes such as *Rhodomonas salina* are vitamin B_{12} auxotrophs and thus could benefit from enhanced vitamins and indirectly support *Dinophysis*. This study in combination with previous work (Hattenrath-Lehmann et al. 2015) demonstrates a strong association between *Dinophysis* and bacteria and suggests it could be based on a requirement for B-vitamins.

Conclusion

This study is the first to demonstrate that *D. acuminata* can assimilate dissolved nitrogen compounds and the first to demonstrate that inorganic and organic nutrients enhance the growth of *D. acuminata* with and without ciliate prey. We emphasize that these findings were obtained using nitrogen levels (50 μmol L^{-1} over 6–12 d) and temperatures that are commonly found within the eutrophic estuaries that host *D. acuminata* blooms (Hattenrath et al. 2010; Hattenrath-Lehmann et al. 2013, 2015), but may be higher than regions hosting blooms in open, non-estuarine systems (Reguera et al. 2012). Additionally, this study, in agreement with Reguera et al. (2012), demonstrated that *Dinophysis* spp. are robust autotrophic survivors, as *D. acuminata* cultures were able to sustain high densities over a 3 week period without the addition of its food source, *M. rubrum*. This can

have important implications for human health as it has been demonstrated that *D. acuminata* retains its toxins as long as cells are viable even when starved (Smith et al. 2012). This work represents the first North American *Dinophysis* strain cultured using prey items isolated from temperate coastal waters throughout the three-step culturing process (i.e., *M. rubrum* and *T. amphioxiea*) and grown at temperatures within the range seen during blooms in North America, thus making these experiments environmentally realistic. Further studies using more strains of *Dinophysis* and prey are needed to better constrain the effects of prey concentration and nutrients on *D. acuminata* growth. In addition, given the new information presented here on bacterial-*Dinophysis* associations, future studies are required to better understand these interactions. Overall, this study suggests that *Dinophysis* blooms can be directly stimulated by N loading and supports the hypothesis that accelerated N loading can promote DSP producing blooms of this species and, thus, may be partly responsible for their recent expansion across North America (Campbell et al. 2010; Hattenrath-Lehmann et al. 2013; Trainer et al. 2013).

References

Berland, B. R., S. Y. Maestrini, D. Grzebyk, and P. Thomas. 1995. Recent aspects of nutrition in the dinoflagellate *Dinophysis cf. acuminata*. Aquat. Microb. Ecol. **9**: 191–198. doi:10.3354/ame009191

Burgess, V., and G. Shaw. 2001. Pectenotoxins—an issue for public health—a review of their comparative toxicology and metabolism. Environ. Int. **27**: 275–283. doi:10.1016/S0160-4120(01)00058-7

Burkholder, J. M., P. M. Glibert, and H. M. Skelton. 2008. Mixotrophy, a major mode of nutrition for harmful algal species in eutrophic waters. Harmful Algae **8**: 77–93. doi:10.1016/j.hal.2008.08.010

Campbell, L., R. J. Olson, H. M. Sosik, A. Abraham, D. W. Henrichs, C. J. Hyatt, and E. J. Buskey. 2010. First harmful *Dinophysis* (Dinophyceae, Dinophysiales) bloom in the U.S. is revealed by automated imaging flow cytometry. J. Phycol. **46**: 66–75. doi:10.1111/j.1529-8817.2009.00791.x

Collos, Y., and P. J. Harrison. 2014. Acclimation and toxicity of high ammonium concentrations to unicellular algae. Mar. Pollut. Bull. **80**: 8–23. doi:10.1016/j.marpolbul.2014.01.006

Crawford, D. W., D. A. Purdie, A. P. M. Lockwood, and P. Weissman. 1997. Recurrent red-tides in the Southampton Water estuary caused by the phototrophic ciliate *Mesodinium rubrum*. Estuar. Coast. Shelf Sci. **45**: 799–812. doi:10.1006/ecss.1997.0242

Croft, M. T., A. D. Lawrence, E. Raux-Deery, M. J. Warren, and A. G. Smith. 2005. Algae acquire vitamin B_{12} through a symbiotic relationship with bacteria. Nature **438**: 90–93. doi:10.1038/nature04056

Deeds, J. R., K. Wiles, G. B. Heideman, K. D. White, and A. Abraham. 2010. First US report of shellfish harvesting closures due to confirmed okadaic acid in Texas Gulf coast oysters. Toxicon **55**: 1138–1146. doi:10.1016/j.toxicon.2010.01.003

Delmas, D., A. Herbland, and S. Y. Maestrini. 1992. Environmental conditions which lead to increase in cell density of the toxic dinoflagellates *Dinophysis* spp. in nutrient-rich and nutrient-poor waters of the French Atlantic coast. Mar. Ecol. Prog. Ser. **89**: 53–61. doi:10.3354/meps089053

Dickey, R. W., G. A. Fryxell, H. R. Granade, and D. Roelke. 1992. Detection of the marine toxins okadaic acid and domoic acid in shellfish and phytoplankton in the Gulf of Mexico. Toxicon **30**: 355–359. doi:10.1016/0041-0101(92)90877-8

Ducklow, H. W. 2000. Bacterial production and biomass in the oceans. In D. L. Kirchman [ed.], Microbial ecology of the Oceans. Wiley.

Freudenthal, A., and J. L. Jijina. 1988. Potential hazards of *Dinophysis* to consumers and shellfisheries. J. Shellfish Res. **7**: 157–158.

Fritz, L., C. G. Stringher, and P. Colepicolo. 1996. Immunolocalization of nitrate reductase in the marine dinoflagellate *Gonyaulax polyedra* (Pyrrophyta). J. Phycol. **32**: 632–637. doi:10.1111/j.0022-3646.1996.00632.x

Fukuda, R., H. Ogawa, T. Nagata, and I. Koike. 1998. Direct determination of carbon and nitrogen contents of natural bacterial assemblages in marine environments. Appl. Environ. Microbiol. **64**: 3352–3358.

Fux, E., J. L. Smith, M. Tong, L. Guzman, and D. M. Anderson. 2011. Toxin profiles of five geographical isolates of *Dinophysis* spp. from North and South America. Toxicon **57**: 275–287. doi:10.1016/j.toxicon.2010.12.002

Giacobbe, M. G., F. Oliva, R. Laferla, A. Puglisi, E. Crisafi, and G. Maimone. 1995. Potentially toxic dinoflagellates in mediterranean waters (Sicily) and related hydrobiological conditions. Aquat. Microb. Ecol. **9**: 63–68. doi:10.3354/ame009063

Glibert, P. M., F. Lipschultz, J. J. Mccarthy, and M. A. Altabet. 1982. Isotope-dilution models of uptake and remineralization of ammonium by marine plankton. Limnol. Oceanogr. **27**: 639–650. doi:10.4319/lo.1982.27.4.0639

Gobler, C. J., A. Burson, F. Koch, Y. Z. Tang, and M. R. Mulholland. 2012. The role of nitrogenous nutrients in the occurrence of harmful algal blooms caused by *Cochlodinium polykrikoides* in New York estuaries (USA). Harmful Algae **17**: 64–74. doi:10.1016/j.hal.2012.03.001

Gobler, C. J., S. Deonarine, J. Leigh-Bell, M. D. Gastrich, O. R. Anderson, and S. W. Wilhelm. 2004. Ecology of phytoplankton communities dominated by *Aureococcus anophagefferens*: The role of viruses, nutrients, and

microzooplankton grazing. Harmful Algae **3**: 471–483. doi:10.1016/j.hal.2004.06.013

Gobler, C. J., and S. A. Sanudo-Wilhelmy. 2003. Cycling of colloidal organic carbon and nitrogen during an estuarine phytoplankton bloom. Limnol. Oceanogr. **48**: 2314–2320. doi:10.4319/lo.2003.48.6.2314

Graneli, E., D. M. Anderson, P. Carlsson, and S. Y. Maestrini. 1997. Light and dark carbon uptake by *Dinophysis* species in comparison to other photosynthetic and heterotrophic dinoflagellates. Aquat. Microb. Ecol. **13**: 177–186. doi:10.3354/ame013177

Guillard, R. R., and J. H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* Cleve. Can. J. Microbiol. **8**: 229–239. doi:10.1139/m62-029

Hackett, J. D., M. Tong, D. M. Kulis, E. Fux, P. Hess, R. Bire, and D. M. Anderson. 2009. DSP toxin production de novo in cultures of *Dinophysis acuminata* (Dinophyceae) from North America. Harmful Algae **8**: 873–879. doi:10.1016/j.hal.2009.04.004

Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia **32**: 79–99. doi:10.2216/i0031-8884-32-2-79.1

Hansen, P. J., M. Moldrup, W. Tarangkoon, L. Garcia-Cuetos, and O. Moestrup. 2012. Direct evidence for symbiont sequestration in the marine red tide ciliate *Mesodinium rubrum*. Aquat. Microb. Ecol. **66**: 63–75. doi:10.3354/ame01559

Hansen, P. J., L. T. Nielsen, M. Johnson, T. Berge, and K. R. Flynn. 2013. Acquired phototrophy in *Mesodinium* and *Dinophysis*—a review of cellular organization, prey selectivity, nutrient uptake and bioenergetics. Harmful Algae **28**: 126–139. doi:10.1016/j.hal.2013.06.004

Hattenrath, T. K., D. M. Anderson, and C. J. Gobler. 2010. The influence of anthropogenic nitrogen loading and meteorological conditions on the dynamics and toxicity of *Alexandrium fundyense* blooms in a New York (USA) estuary. Harmful Algae **9**: 402–412. doi:10.1016/j.hal.2010.02.003

Hattenrath-Lehmann, T. K., M. A. Marcoval, D. L. Berry, S. Fire, Z. Wang, S. L. Morton, and C. J. Gobler. 2013. The emergence of *Dinophysis acuminata* blooms and DSP toxins in shellfish in New York waters. Harmful Algae **26**: 33–44. doi:10.1016/j.hal.2013.03.005

Hattenrath-Lehmann, T. K., and others. 2015. Nitrogenous nutrients promote the growth and toxicity of *Dinophysis acuminata* during estuarine bloom events. PLoS One **10**: e0124148. doi:10.1371/journal.pone.0124148

Herfort, L., and others. 2012. Red waters of *Myrionecta rubra* are biogeochemical hotspots for the Columbia River estuary with impacts on primary/secondary productions and nutrient cycles. Estuaries Coasts **35**: 878–891. doi:10.1007/s12237-012-9485-z

Horner, R. A., D. L. Garrison, and F. G. Plumley. 1997. Harmful algal blooms and red tide problems on the U. S. west coast. Limnol. Oceanogr. **42**: 1076–1088. doi:10.4319/lo.1997.42.5_part_2.1076

Jeong, H. J., and others. 2005. Feeding by phototrophic red-tide dinoflagellates: Five species newly revealed and six species previously known to be mixotrophic. Aquat. Microb. Ecol. **40**: 133–150. doi:10.3354/ame040133

Jochem, F. J. 2001. Morphology and DNA content of bacterioplankton in the northern Gulf of Mexico: Analysis by epifluorescence microscopy and flow cytometry. Aquat. Microb. Ecol. **25**: 179–194. doi:10.3354/ame025179

Johansson, N., E. Graneli, T. Yasumoto, P. Carlsson, and C. Legrand. 1996. Toxin production by *Dinophysis acuminata* and *D. acuta* cells grown under nutrient sufficient and deficient conditions, p. 277-280. In T. Yasumoto, Y. Oshima and Y. Fukuyo [eds.], The Seventh International Conference on Toxic Phytoplankton. Intergovernmental Oceanographic Commission of UNESCO.

Jones, M. N. 1984. Nitrate reduction by shaking with cadmium—alternative to cadmium columns. Water Res. **18**: 643–646. doi:10.1016/0043-1354(84)90215-X

Kamiyama, T., and T. Suzuki. 2009. Production of dinophysistoxin-1 and pectenotoxin-2 by a culture of *Dinophysis acuminata* (Dinophyceae). Harmful Algae **8**: 312–317. doi:10.1016/j.hal.2008.07.003

Kazamia, E., and others. 2012. Mutualistic interactions between vitamin B_{12}-dependent algae and heterotrophic bacteria exhibit regulation. Environ. Microbiol. **14**: 1466–1476. doi:10.1111/j.1462-2920.2012.02733.x

Kim, S., Y. G. Kang, H. S. Kim, W. Yih, D. W. Coats, and M. G. Park. 2008. Growth and grazing responses of the mixotrophic dinoflagellate *Dinophysis acuminata* as functions of light intensity and prey concentration. Aquat. Microb. Ecol. **51**: 301–310. doi:10.3354/ame01203

Kirchman, D. L. 2008. Microbial ecology of the Oceans, 2nd ed. Wiley.

Koch, F., T. K. Hattenrath-Lehmann, J. A. Goleski, S. Sañudo-Wilhelmy, N. S. Fisher, and C. J. Gobler. 2012. Vitamin B_1 and B_{12} uptake and cycling by plankton communities in coastal ecosystems. Front. Microbiol. **3**: 363. doi:10.3389/fmicb.2012.00363

Koch, F., S. A. Sanudo-Wilhelmy, N. S. Fisher, and C. J. Gobler. 2013. Effect of vitamins B_1 and B_{12} on bloom dynamics of the harmful brown tide alga, *Aureococcus anophagefferens* (Pelagophyceae). Limnol. Oceanogr. **58**: 1761–1774. doi:10.4319/lo.2013.58.5.1761

Koukaras, K., and G. Nikolaidis. 2004. *Dinophysis* blooms in Greek coastal waters (Thermaikos Gulf, NW Aegean Sea). J. Plankton Res. **26**: 445–457. doi:10.1093/plankt/fbh042

Lee, J.-S., T. Igarashi, S. Fraga, E. Dahl, P. Hovgaard, and T. Yasumoto. 1989. Determination of diarrhetic shellfish toxins in various dinoflagellate species. J. Appl. Phycol. **1**: 147–152. doi:10.1007/BF00003877

Lee, S., Y. C. Kang, and J. A. Fuhrman. 1995. Imperfect retention of natural bacterioplankton cells by glass-fiber

filters. Mar. Ecol. Prog. Ser. **119**: 285–290. doi:10.3354/meps119285

Lucas, I. A. N., and M. Vesk. 1990. The fine structure of two photosynthetic species of *Dinophysis* (Dinophysiales, Dinophyceae). J. Phycol. **26**: 345–357. doi:10.1111/j.0022-3646.1990.00345.x

Minnhagen, S., and S. Janson. 2006. Genetic analyses of *Dinophysis* spp. support kleptoplastidy. FEMS Microbiol. Ecol. **57**: 47–54. doi:10.1111/j.1574-6941.2006.00096.x

Minnhagen, S., M. Kim, P. S. Salomon, W. Yih, E. Graneli, and M. G. Park. 2011. Active uptake of kleptoplastids by *Dinophysis caudata* from its ciliate prey *Myrionecta rubra*. Aquat. Microb. Ecol. **62**: 99–108. doi:10.3354/ame01459

Montoya, J. P., M. Voss, P. Kahler, and D. G. Capone. 1996. A simple, high-precision, high-sensitivity tracer assay for N_2 fixation. Appl. Environ. Microbiol. **62**: 986–993.

Myung, G., H. S. Kim, J. W. Park, J. S. Park, and W. Yih. 2013. Sequestered plastids in *Mesodinium rubrum* are functionally active up to 80 days of phototrophic growth without cryptomonad prey. Harmful Algae **27**: 82–87. doi:10.1016/j.hal.2013.05.001

Nagai, S., T. Suzuki, T. Nishikawa, and T. Kamiyama. 2011. Differences in the production and excretion kinetics of okadaic acid, dinophysistoxin-1, and pectenotoxin-2 between cultures of *Dinophysis acuminata* and *Dinophysis fortii* isolated from western Japan. J. Phycol. **47**: 1326–1337. doi:10.1111/j.1529-8817.2011.01076.x

Nielsen, L. T., B. Krock, and P. J. Hansen. 2012. Effects of light and food availability on toxin production, growth and photosynthesis in *Dinophysis acuminata*. Mar. Ecol. Prog. Ser. **471**: 37–50. doi:10.3354/meps10027

Nielsen, L. T., B. Krock, and P. J. Hansen. 2013. Production and excretion of okadaic acid, pectenotoxin-2 and a novel dinophysistoxin from the DSP-causing marine dinoflagellate *Dinophysis acuta*—effects of light, food availability and growth phase. Harmful Algae **23**: 34–45. doi:10.1016/j.hal.2012.12.004

Orcutt, K. M., F. Lipschultz, K. Gundersen, R. Arimoto, A. F. Michaels, A. H. Knap, and J. R. Gallon. 2001. A seasonal study of the significance of N_2 fixation by *Trichodesmium* spp. at the Bermuda Atlantic Time-series Study (BATS) site. Deep-Sea Res. Part II Top. Stud. Oceanogr. **48**: 1583–1608. doi:10.1016/S0967-0645(00)00157-0

Park, M. G., S. Kim, H. S. Kim, G. Myung, Y. G. Kang, and W. Yih. 2006. First successful culture of the marine dinoflagellate *Dinophysis acuminata*. Aquat. Microb. Ecol. **45**: 101–106. doi:10.3354/ame045101

Parsons, T. R., Y. Maita, and C. M. Lalli. 1984. A manual of chemical and biological methods for seawater analysis. Pergamon Press.

Raho, N., G. Pizarro, L. Escalera, B. Reguera, and I. Marin. 2008. Morphology, toxin composition and molecular analysis of *Dinophysis ovum* Schutt, a dinoflagellate of the "*Dinophysis acuminata* complex." Harmful Algae **7**: 839–848. doi:10.1016/j.hal.2008.04.006

Raux, E., H. L. Schubert, and M. J. Warren. 2000. Biosynthesis of cobalamin (vitamin B_{12}): A bacterial conundrum. Cell. Mol. Life Sci. **57**: 1880–1893. doi:10.1007/PL00000670

Reguera, B., L. Velo-Suarez, R. Raine, and M. G. Park. 2012. Harmful *Dinophysis* species: A review. Harmful Algae **14**: 87–106. doi:10.1016/j.hal.2011.10.016

Riisgaard, K., and P. J. Hansen. 2009. Role of food uptake for photosynthesis, growth and survival of the mixotrophic dinoflagellate *Dinophysis acuminata*. Mar. Ecol. Prog. Ser. **381**: 51–62. doi:10.3354/meps07953

Seeyave, S., T. A. Probyn, G. C. Pitcher, M. I. Lucas, and D. A. Purdie. 2009. Nitrogen nutrition in assemblages dominated by *Pseudo-nitzschia* spp., *Alexandrium catenella* and *Dinophysis acuminata* off the west coast of South Africa. Mar. Ecol. Prog. Ser. **379**: 91–107. doi:10.3354/meps07898

Smith, J. L., M. M. Tong, E. Fux, and D. M. Anderson. 2012. Toxin production, retention, and extracellular release by *Dinophysis acuminata* during extended stationary phase and culture decline. Harmful Algae **19**: 125–132. doi:10.1016/j.hal.2012.06.008

Stoecker, D. K., U. Tillmann, and E. Graneli. 2006. Phagotrophy in harmful algae, p. 177-187. In E. Graneli and J. T. Turner [eds.], Ecology of harmful algae, ecological studies. Springer.

Swanson, K. M., L. J. Flewelling, M. Byrd, A. Nunez, and T. A. Villareal. 2010. The 2008 Texas *Dinophysis ovum* bloom: Distribution and toxicity. Harmful Algae **9**: 190–199. doi:10.1016/j.hal.2009.10.001

Tang, Y. Z., F. Koch, and C. J. Gobler. 2010. Most harmful algal bloom species are vitamin B_1 and B_{12} auxotrophs. Proc. Natl. Acad. Sci. **107**: 20756–20761. doi:10.1073/pnas.1009566107

Tong, M., D. M. Kulis, E. Fux, J. L. Smith, P. Hess, Q. Zhou, and D. M. Anderson. 2011. The effects of growth phase and light intensity on toxin production by *Dinophysis acuminata* from the northeastern United States. Harmful Algae **10**: 254–264. doi:10.1016/j.hal.2010.10.005

Tong, M., Q. Zhou, M. D. Kulis, T. Jiang, Y. Qi, and M. D. Anderson. 2010. Culture techniques and growth characteristics of *Dinophysis acuminata* and its prey. Chin. J. Oceanol. Limnol. **28**: 1230–1239. doi:10.1007/s00343-010-9960-y

Tong, M. M., J. L. Smith, D. M. Kulis, and D. M. Anderson. 2013. The food quality and dissolved inorganic nutrient effect on the growth and toxicity of *Dinophysis acuminata* from North America, p. 120. Seventh symposium on harmful algae in the U.S.

Trainer, V., and others. 2013. Diarrhetic shellfish toxins and other lipophilic toxins of human health concern in

Washington State. Mar. Drugs **11**: 1815–1835. doi: 10.3390/md11061815

Van Dolah, F. M. 2000. Marine algal toxins: Origins, health effects, and their increased occurrence. Environ. Health Perspect. **108**: 133–141. doi:10.1289/ehp.00108s1133

Wilkerson, F. P., and G. Grunseich. 1990. Formation of blooms by the symbiotic ciliate *Mesodinium rubrum*—the significance of nitrogen uptake. J. Plankton Res. **12**: 973–989. doi:10.1093/plankt/12.5.973

Wisecaver, J. H., and J. D. Hackett. 2010. Transcriptome analysis reveals nuclear-encoded proteins for the maintenance of temporary plastids in the dinoflagellate *Dinophysis acuminata*. BMC Genomics **11**: 366. doi:10.1186/1471-2164-11-366

Acknowledgments

We gratefully acknowledge useful comments and perspective provided by two anonymous reviewers. Thank you to M. Lusty and Y. Kang for assistance with experiments. This is MERHAB contribution #183. We are grateful for financial support from NOAA's Monitoring and Event Response to Harmful Algal Blooms (MERHAB) program (NA11NOS4780027) and New York Sea Grant (R/CMB-38-NYCT). We are sincerely grateful to P.J. Hansen and L.T. Nielsen for generously providing us with cultures of *M. rubrum* and *T. amphioxeia* and R. Kudela for ^{15}N analysis.

Submitted 15 November 2014
Revised 2 March 2015; 6 May 2015
Accepted 8 May 2015

Associate editor: Heidi Sosik